0041-1337/98/6610-1272$03.00/0
TRANSPLANTATION

Vol. 66, 1272–1278, No. 10, November 27, 1998
Printed in U.S.A.

REPAIR OF CRANIOTOMY DEFECTS USING BONE MARROW STROMAL CELLS

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Background. **Techniques used to repair craniofacial skeletal defects parallel the accepted surgical therapies for bone loss elsewhere in the skeleton and include the use of autogenous bone and alloplastic materials. Transplantation of a bone marrow stromal cell population that contains osteogenic progenitor cells may be an additional modality for the generation of new bone.**

Methods. **Full thickness osseous defects (5 mm) were prepared in the cranium of immunocompromised mice and were treated with gelatin sponges containing murine alloplastic bone marrow stromal cells derived from transgenic mice carrying a type I collagen-chloramphenicol acetyltransferase reporter gene to follow the fate of the transplanted cells. Control surgical sites were treated with spleen stromal cells or gelatin sponges alone, or were left untreated. The surgical defects were analyzed histologically for percent closure of the defect at 2, 3, 4, 6, and 12 weeks.**

Results. **Cultured bone marrow stromal cells transplanted within gelatin sponges resulted in osteogenesis that repaired greater than 99.0±2.20% of the original surgical defect within 2 weeks. In contrast, cranial defects treated with splenic fibroblasts, vehicle alone, or sham-operated controls resulted in minimal repair that was limited to the surgical margins. Bone marrow**

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stromal cells carrying the collagen transgene were immunodetected only in the newly formed bone and thus confirmed the donor origin of the transplanted cells.

Conclusions. **These studies demonstrate that mitotically expanded bone marrow cells can serve as an abundant source of osteoprogenitor cells that are capable of repairing craniofacial skeletal defects in mice without the addition of growth or morphogenetic factors.**

Persistent defects in craniofacial bones present a formidable surgical challenge and are a subject of active clinical concern. Although most often secondary to trauma, craniofacial osseous deficiencies can also arise from infection, resection of tumors, congenital malformations, and progressive deforming skeletal diseases (*1–4*). In addition to leaving the patient with an aesthetic deformity, such defects may be uncomfortable to the patient and directly affect function. Techniques to repair these defects parallel the accepted surgical therapies for bone loss elsewhere in the skeleton and include the use of autologous bone and alloplastic material. Bone autografts can include cranial bone, split-thickness rib, iliac crest cortical or cancellous bone, and split-thickness tibial bone. However, each of these techniques suffers from the limited availability of donor tissue, potentially debilitating donor site discomfort, and variable rates of resorption, which may influence graft success (*5–7*).

To overcome some of the limitations of autografts, cadaveric allografts in the form of demineralized bone matrix have also been used to induce new bone formation (*8*). The clinical utility of these materials is attributed to the presumed bone inductive activities of bone morphogenetic proteins (BMPs*) present in the allograft (*9–11*). Although a number of studies report the clinical success of demineralized bone allografts in repairing osseous defects, others have reported inconsistent bone inductive activities that vary widely between tissue banks and even among different lots from the same source (*12, 13*). Therefore, allograft bone continues to have a limited utility for closing defects and continues to carry the risk of transmitting pathogens from host to recipient.

Alloplastic bone substitutes, including stainless steel mesh, titanium mesh, and methylmethacrylate paste, are widely available and are relatively easy to apply to osseous defects. Alloplastic ceramics in the form of hydroxyapatite blocks or granules also serve as effective space fillers that differ in both their handling characteristics and structural integrity (*14, 15*). These osteoconductive materials provide a rigid scaffold to facilitate cell adhesion and eventual migration of bone forming cells into the defect site. Nonceramic hydroxyapatite bone cement can be shaped at the time of surgery and may be useful in many anatomic locations (*16*). The major limitations of these alloplastic materials, however, are that they are slow to resorb (*17*) and are often surrounded by a connective tissue capsule that may affect graft stability (*18*). Consequently, an ideal material or method for reconstructing craniofacial defects has yet to be developed.

A potential method for skeletal regeneration that would permit the use of autogenous cells without the addition of exogenous growth or morphogenetic factors is the utilization of in vitro expanded bone marrow stromal cells (BMSCs). The presence of a bone marrow-derived stromal cell population with osteogenic potential was initially demonstrated by Friedenstein (*19*), and further characterized by others (*20–22*). These cells are strongly adherent to the substrate in tissue culture flasks and can be distinguished from hematopoietic cells and macrophages by a number of morphological, histochemical, and biochemical characteristics (*23*). Isolation of relatively pure BMSC cultures can be attained after subculture; however, the degree of purification may be dependent on the species and strain of animal used. Contamination with hematopoietic cells is higher in mouse BMSC strains than human BMSCs, and is diminished during consecutive passages in vitro (*24, 25*). In addition to their fibroblast-like morphology, BMSCs share some, but not all, features with fibroblastic cells of other tissues, and lack the basic characteristics of endothelial cells and macrophages (*26–29*). Cultured BMSCs maintain many of the multipotential, differentiative features that define a stem cell, i.e., they are capable of self-renewal and they can differentiate into several phenotypes including bone (*30, 31*). Thus, in vitro expanded BMSCs may be a rich source of osteogenic progenitor cells that are capable of promoting the repair or regeneration of craniofacial osseous defects.

The development of therapies to accelerate bone formation in skeletal defects using autologous cells may circumvent many of the limitations of auto- and allografting methods. The mitotically expanded BMSCs can serve as an abundant source of osteoprogenitor cells that maintain osteogenic properties when transplanted. We and others have developed in vitro culture methods to expand marrow stromal cells derived from mouse and human bone marrow aspirates, and have shown that these cells are capable of forming new bone in an in vivo animal model within a number of different vehicles (*24, 25, 32, 33*). The aim of this investigation was to determine if cultured BMSCs could close craniotomy defects in mice without the addition of exogenous growth or morphogenetic factors. In this study, we demonstrate that cultured BMSCs transplanted within gelatin sponges are capable of rapid and consistent regeneration of critical-size cranial defects in mice.

MATERIALS AND METHODS

Source of bone marrow cells. Eight- to 14-week-old transgenic mice carrying rat α1(I) procollagen-chloramphenicol acetyltransferase (CAT) constructs were used to harvest bone marrow (*34*). All procedures were performed in accordance to specifications of an approved small animal protocol (114–93). Bone marrow from the femoral, tibial, and humeral medullary cavities was flushed with α-modified minimum essential medium (αMEM, Life Technologies, Inc., Grand Island, NY). The entire marrow content of six bones (two each of femur, tibia, and humerus), which contained $6-8\times10^7$ nucleated cells, was plated into a 75-cm^2 culture flask (Becton Dickinson, Franklin Lakes, NJ) in 30 ml of growth medium (αMEM, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate [Biofluids, Rockville, MD], and 20% fetal bovine serum [Life Technologies, or Atlanta Biological, Norcross, GA]). Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Confluent layers of adherent cells were formed by 11–14 days. The adherent layers were harvested using the following protocol: (1) two washes with Hanks' balanced salt solution (Life Technologies), (2) incubation with chondroitinase ABC (20 mU/ml, Seidaguku Corp., Tokyo, Japan) in αMEM for 25–35 min at 37°C, (3) one wash with Hanks'

* Abbreviations: αMEM, α-modified minimum essential medium; BMP, bone morphogenetic protein; BMSC, bone marrow stromal cell; CAT, chloramphenicol acetyltransferase.

balanced salt solution, (4) incubation with 1× trypsin-EDTA (Life Technologies) for 25–35 min at room temperature, (5) a second incubation with trypsin-EDTA for 25–35 min at 37°C, and (6) a final wash in growth medium. The subcultured cells were plated at 2×10^6 cells/75-cm^2 flask. Steps 2 and 3 were omitted after the second subculture of cells. Marrow cells were centrifuged at 1000 rpm for 10 min, and the cell pellet was resuspended in fresh αMEM containing 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies) at 37°C and 5% CO_2

Loading cells into gelatin sponges. Between 3.0 and 5.0×10^6 mouse BMSCs from passage 2–10 were transplanted per gelatin sponge (Gelfoam™, Upjohn, Kalamazoo, MI). The cells were harvested by trypsin-EDTA treatment and were resuspended in 30–100 μl of growth medium. Approximately 25–50-mm^3 gelatin sponge sheets were preincubated in the culture medium for 15 min. Air bubbles were removed by gently pressing the sponges with forceps. Immediately before loading, the sponges were dried by pressing them between two sheets of sterile filter paper, and vehicles were then immediately placed into the cell suspension where they expanded and took up the cells. In separate experiments, direct cell counts (Coulter counter, model ZBI; Coulter Electonics, Hialeah, FL) of the residual suspension after removal of the gelatin sponge showed that greater than 95% of the cells entered the sponges. The percentage of cells that did not enter the sponge was 2.99±1.88 (n=12). After loading the sponges with the cells, all vehicles were incubated at 37°C for 30 min before transplantation.

Recipient mice with craniotomy defects. NIH-BG-NU-XID immunocompromised mice (6–10-week-old females) were used as transplantation recipients. Operations were performed under anesthesia achieved by an intraperitoneal injection of 2.5% tribromoethanol (Sigma Chemical, St. Louis, MO) at a dosage of 0.2 ml/kg of body weight. One midline skin incision of approximately 1 cm in length was made on the dorsal surface of the cranium, and the skin and periosteum were separated by blunt dissection. A 5-mm cranial defect was created with a trephine bur (Fine Science Tools Inc., Foster City, CA) attached to an electric Dremell handpiece (Dremell, Racine, WI). A full-thickness calvarial bone defect was prepared with minimal invasion of the dura mater. Gelatin sponges with or without BMSCs were placed over the cranial defect, and the skin was sutured with 5–0 Vicryl suture (Ethicon™, Johnson and Johnson Co., Somerville, NJ). Animals in which cranial defects were created but which received no implant served as sham-operated controls. The distribution of surgical sites evaluated for histologic analysis is listed in Table 1. Craniotomy defects in mice were divided into four treatment groups, which included defects left unfilled, defects filled with a gelatin sponge alone, defects filled with a gelatin sponge seeded with mouse spleen cells, or defects filled with a gelatin sponge seeded with mouse BMSCs. A total of 49 implant experiments were performed, with postsurgical analysis completed at times ranging from 2 to 12 weeks.

Preparation of spleen stromal cells. Spleen stromal cells served as an additional control and were chosen as a convenient source of nonosteogenic, primary cells. Cell suspensions from mouse spleens were prepared by dissecting the spleen parenchyma and mincing the tissue in αMEM. Between 10 and 20×10^7 nucleated spleen cells were plated into 30 ml of growth medium in 75-cm^2 flasks. After the adherent layer approached confluence, the cells were subcultured using the same technique as described for the marrow cultures. Between 4 and 5×10^6 spleen stromal cells from passage number 2 to 3 were transplanted into the craniotomy defects within gelatin sponges.

Fixation and histological examination of the transplants. The calvariae were dissected at 2–12 weeks after transplantation and were fixed and partially decalcified for 2 days in Bouin's solution (Sigma Chemical). The transplants were embedded in paraffin after dehydration with ascending concentrations of ethanol and xylene. Paraffin sections (5 μm) were deparaffinized, hydrated, and stained with hemotoxylin and eosin. Determination of the percentage of the craniotomy defect that was closed was performed using digitized microscopy and NIH Image software.

Immunohistochemistry. The dissected calvariae were fixed overnight with freshly prepared 4% paraformaldehyde in phosphate-buffered saline at 4°C and were decalcified with 10% EDTA (pH 8.0) at 4°C for 2 days. The calvariae were embedded in OCT compound (Miles, Elkhart, IN), frozen rapidly in ethanol-dry ice, and sectioned with a frozen microtome. Frozen sections of 7–10 μm in thickness were immersed in phosphate-buffered saline for 10 min. Endogenous peroxidase was inactivated by incubation with 3% H_2O_2 for 30 min. An avidin-biotinylated peroxidase complex was completed using rabbit anti-CAT antibody at a 1:500 dilution (5Prime-3Prime Inc., Boulder, CO) as the primary antibody, and biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) as the second antibody. The immunolocalized antigen was visualized by diaminobenzidine (HistoMark; Kirkegaard and Perry Laboratories). Normal rabbit serum (1:500) and normal rabbit IgG (17 μg/ml) served as negative controls.

Statistical analysis. An unpaired *t* test was used to determine any difference from the control and experimental group where only two time points are compared (2, 3, and 12 weeks). A repeated-measures analysis of variance using the Bonferroni post hoc correction test was used when more than two means were compared at a single time point (4 and 6 weeks). All reported values are means (X) ± standard deviation (SD); $P<0.05$ was considered statistically significant.

TABLE 1. Number of implants per treatment group

Transplants	2 week	3 weeks	4 weeks	6 weeks	12 weeks
BMSCs+gelatin[a]	5	4	7	6	3
Control 1[b]	—[c]	—	2	3	4
Control 2[d]	3	2	3	3	—
Spleen stromal cells+gelatin	1	—	3	—	—

[a] Gelfoam sponge (Upjohn Co., Kalamazoo, MI).
[b] Sham-operated control; no gelatin sponge or cells added.
[c] —, Not done.
[d] Gelatin sponge without BMSCs.

RESULTS

Osseous regeneration with BMSCs. BMSCs were capable of osteogenesis leading to closure of the cranial defects as early as 2 weeks after transplantation. Representative examples of calvarial regeneration by BMSCs are shown in Figure 1. Closure of the defect with new bone surrounded by a periosteal layer was observed in all transplants of BMSCs and was nearly complete by the second postoperative week (Fig. 1A). The thickness of the newly reconstructed defect was variable and seemed to be related to the thickness of the gelatin sponge. The gelatin sponges were carved by hand to approximate the defect, and the thickness was often larger than the defect to allow a greater number of cells to seed the sponge. Where a thick sponge was used, the newly formed bone had a more pronounced marrow space observed at the time of histological analysis (Fig. 1C). However, this size discrepancy was not appreciable macroscopically. In a few experiments, the new bone appeared to be fused to the marginal bone of the recipient calvarium. In most cases, however, the newly formed bone overlapped the margins of the recipient site (Fig. 1D). In one experiment, a minimal closure of 55.0% of the original defect was observed at 6 weeks of healing with BMSCs within a gelatin sponge (Table 2). The majority of experiments with BMSCs led to rapid and nearly

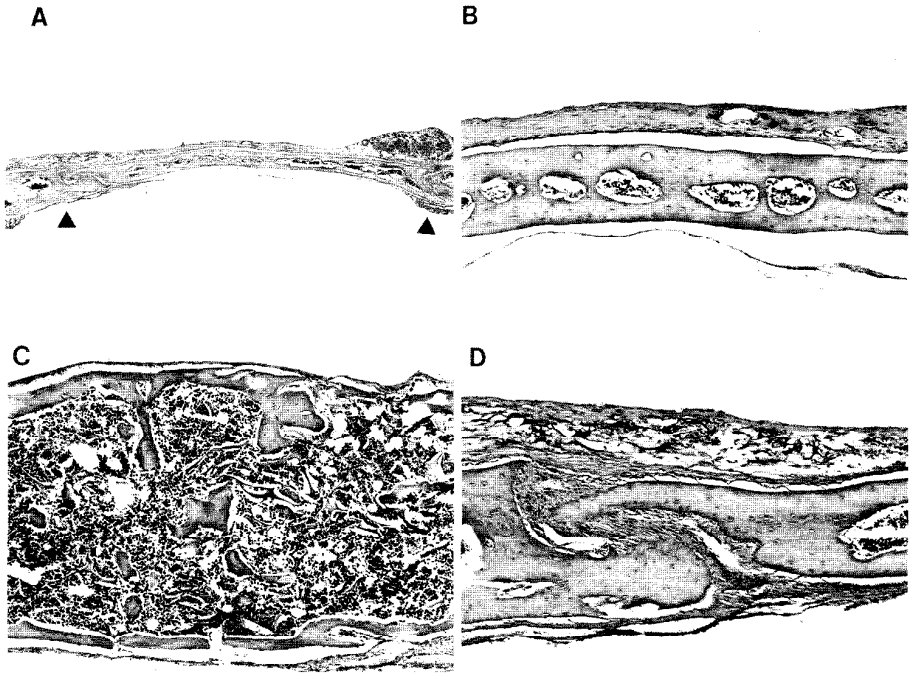

FIGURE 1. Repair of craniotomy defects with BMSCs. (A) Cranial defect closure with mouse BMSCs seeded within a gelatin sponge approximating the size of the defect 2 weeks after transplantation (original magnification, ×25). (B) Cranial defect closure with mouse BMSCs seeded within a gelatin sponge carved to approximate the defect (original magnification, ×100). (C) Cranial defect closure with mouse BMSCs seeded within a gelatin sponge carved thicker than the defect to allow a greater number of cells to be transplanted (original magnification, ×100). (D) Margin of repaired craniotomy defect (original magnification, ×100). The arrowheads designate the surgical margins.

TABLE 2. Osteogenesis by BMSCs in craniotomy defects (numbers are percent of defect repaired)

Transplantation procedure	2 weeks		3 weeks		4 weeks		6 weeks		12 weeks	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
BMSCs + gelatin[a]	99.0±2.02[d]	95.1–100	94.1±6.3[d]	88.2–100	99.1±1.7[d]	97.1–100	92.5[d]±18.4	55.0–100	100[d]	100–100
Control 1[b]	ND[e]		ND		3.7±6.4	0–11.0	3.3±5.2	0–9.0	6.7±5.8	0–11.0
Control 2[c]	3.9±6.7	0.0–11.7	0.0		0.0		4.0±4.0	0–0.8	ND	
Spleen stromal cells + gelatin	17.7		ND		14.8±25.6[d]	0–44.4	ND		ND	

[a] Gelfoam sponge (Upjohn Co., Kalamazoo, MI).
[b] Sham-operated control; no gelatin sponge or cells added.
[c] Gelatin sponge without BMSCs.
[d] Different from controls, $P<0.05$.
[e] ND, not done.

complete regeneration of the defect with a mean percent closure of 92.5–100%.

In contrast, bridging of the cranial defects with new bone was not a significant feature in animals receiving either a gelatin sponge alone or a gelatin sponge loaded with spleen stromal cells. In the few instances when osteogenesis was observed in sham-operated surgical sites, new bone formation was limited to the margins of the defect. These control experiments were analyzed after 4–12 weeks of healing. The mean percentage defect repaired by new bone ranged from 3.3% to 6.7%, with a maximal closure of 11.0% (Table 2). The healing response in this control group included only a thin layer of connective tissue spanning the defect (Fig. 2A). In defects treated with either a gelatin sponge alone or a gelatin sponge seeded with spleen stromal cells, histologic evidence of healing included a thicker connective tissue layer surrounding remnants of the resorbing gelatin vehicle (Fig. 2, B and C). In one experiment that included spleen stromal cells within a gelatin sponge, new bone closed about 44% of the craniotomy defect after 4 weeks of healing. However, in all other control experiments using spleen cells, repair of the defects with new bone was never greater than 18% of the original defect (Table 2).

Determination of osteoblast origin. BMSCs derived from transgenic mice carrying 3.6 kilobases of the rat $\alpha(1)$ I collagen promoter fused to the CAT reporter gene were used to follow the fate of the transplanted cells. Because the reporter gene is not present in the recipient cells, CAT expression served as a marker for donor cell activity. Immunostaining of tissue sections with an antibody raised against CAT was detected in osteoblasts and osteocytes within the new bone, confirming that the osteogenic cells were of donor origin rather than those of the local microenvironment (Fig. 3). CAT immunoreactivity was restricted to the new bone, suggesting that the BMSCs did not migrate into the surrounding tissues. CAT immunoreactivity was not observed with either the nonimmune or normal rabbit IgG negative controls (data not shown). No signs of inflammation or dysplasia were detected in the peritransplant region of any of the transplants analyzed.

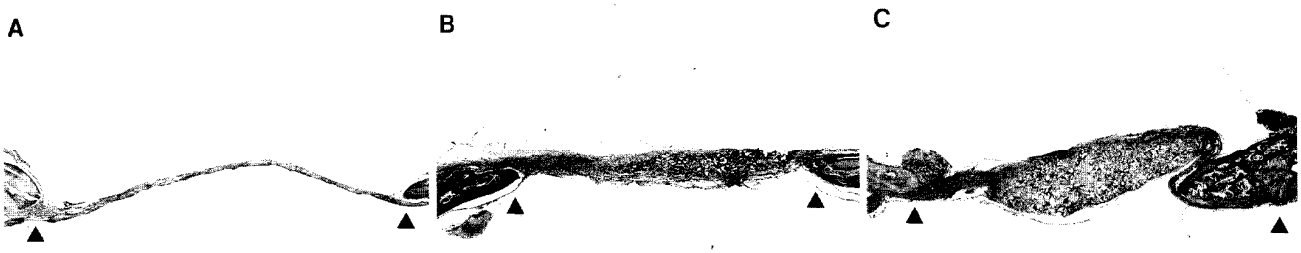

FIGURE 2. Healing of craniotomy defects without the addition of BMSCs (original magnification, ×25). (A) A thin layer of connective tissue spans the sham operated cranial defect 4 weeks after transplantation. Minimal bone formation was observed only at the surgical margins. (B) Cranial defect healing with a gelatin sponge alone 4 weeks after transplantation. (C) Cranial defect healing with spleen fibroblasts seeded within a gelatin sponge 4 weeks after transplantation. The arrowheads designate the surgical margins.

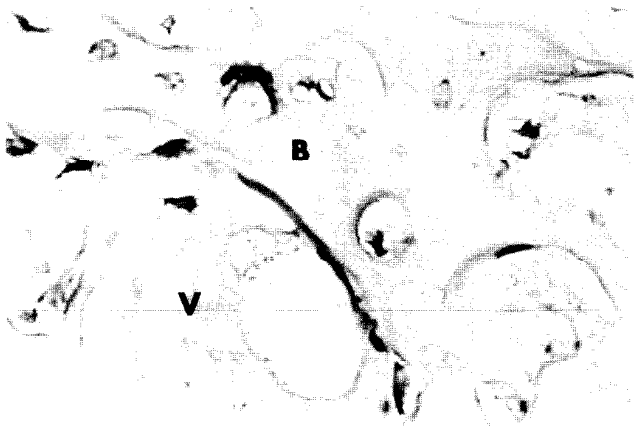

FIGURE 3. Confirmation of the donor origin of newly formed bone (original magnification, ×400). Cranial defects were treated with a gelatin sponge-containing mouse BMSCs derived from transgenic mice carrying the ColCAT3.6 transgene. Immunoreactivity for CAT is localized to osteoblasts and osteocytes in the regenerated cranial defect showing the donor origin of the new bone. B is newly formed bone. V is the resorbing vehicle.

DISCUSSION

The repair of critical size defects is a well-established model system for investigating the efficacy of biomaterials and cell signaling factors in inducing new bone formation (*35*). Cranial defects have been successfully closed by combining recombinant BMPs with hydroxyapatite and collagen carriers (*36–39*), demineralized bone matrix (*40*), and bioresorbable polymer substrates (*41, 42*). Similar defects have also been closed using the principles of guided tissue regeneration to selectively promote the growth and differentiation of osteoprogenitor cells (*43*). Transplantation of a BMSC population that contains osteoblastic precursors offers an additional modality for the generation of new bone.

Mouse BMSCs generated by the method employed in this study consist predominantly of the progeny of single precursor cells termed colony-forming unit-fibroblastic cells (*44*). The proportion of hematopoietic cells (primarily macrophages) in these strains is reduced from about 11% of the total cell number at passage number 2, to less than 5% at passage 10 (*24*). Here, we demonstrate that cultured mouse BMSCs have the capacity to repair craniotomy defects without the addition of exogenous growth or morphogenetic factors. Osseous defects that do not heal within the lifetime of the animal have been termed critical size defects (*35*). Such critical size defects have been demonstrated in the rat, rabbit, dog, and nonhuman primates (*45, 46*). Although our studies were not designed to formally determine critical size craniotomy defects in mice, the sham-operated defects and defects treated with the gelatin vehicle alone showed minimal healing that was restricted to the surgical margins after 12 weeks of healing. The defects treated with BMSCs were closed as early as the second postoperative week, and the newly formed bone was determined to be of donor origin by identifying immunoreactive CAT expressed exclusively by the donor cells.

The possibility exists that BMSCs may migrate to distant sites when delivered via an intravenous route. When immortalized marrow cells carrying a human minigene for collagen I was introduced intravenously, a sensitive polymerase chain reaction method was able to detect the transgene in a number of tissues after 1–5 months (*47*). Other groups, however, have demonstrated that marrow stromal cells do not migrate to bone or bone marrow after intravenous injection (*48, 49*). The failure to identify BMSCs migrating to the local microenvironment in this study may be a result of differences in the sensitivities of detection methods. The transplantability of marrow stromal cells by injection remains controversial and must be addressed before predictable gene- or cell-based tissue replacement therapies via this route can be realized.

To determine whether BMSCs may have clinical utility, larger animal models must be evaluated to confirm that larger volume transplants can still form adequate bone with the appropriate functional integrity. The creation of larger transplants would also allow for meaningful biomechanical studies. Ideally, a larger animal would permit the use of autologous cells. Additional studies would also establish the longevity of the transplants, the optimum cell to matrix ratio, and the minimum numbers of cells needed per volume of defect. However, it should be noted that the requirements for both the ex vivo expansion and vehicle composition may vary between species (*24*).

Because of the many complex skeletal defects that can present clinically, it is unlikely that a single molecule, graft, or substrate will be successful in the regeneration of bone in

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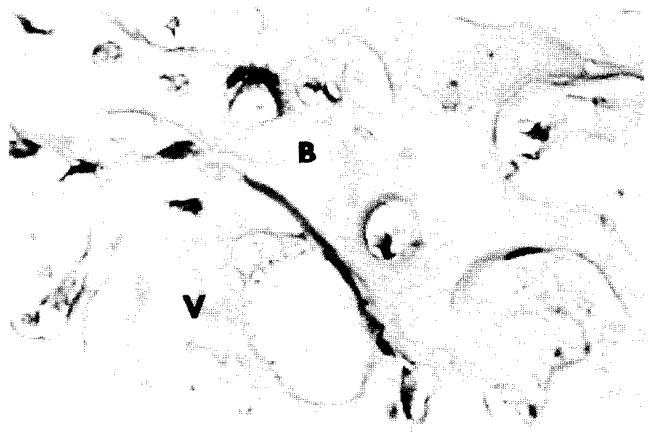

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Received 6 May 1998.
Accepted 11 August 1998.